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(54) Title: INSULIN ANALOGUES (57) Abstract Novel insulin analogues are provided in which one or more of the amino acid residues in position A18, A21 and B3 are different from Asn and/or one or more of the amino acid residues in position A5, A15 and B4 are different from Gln.		

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INSULIN ANALOGUES

Technical Field

5 The present invention relates to novel insulin analogues being stabilized against chemical modifications and to novel insulin preparations containing such insulin analogues.

10 Background of the invention

When insulin is handled at body - (or even room -) temperature the biological potency tends to decline as a function of time. Such loss of biological potency may be
15 ascribed certain chemical modifications of insulin occurring during storage.

Like other proteins, insulin is not a stable entity but is liable to degradation by chemical reactions with molecules
20 or ions in its vicinity, or to intra- and intermolecular transformations within the insulin molecules.

Hydrolytic transformation of insulin in acid solution with the liberation of ammonium ions and formation of
25 deamidation products has been reported by Sundby (J.Biol.Chem. 237 (1962), 3406-4311) who demonstrated that monodesamido-(A21)-insulin is the prevailing derivative formed.

30 Jackson et al. (Diabetes 21 (1972), 235-245) compared acid and neutral solutions and showed that similar, but less pronounced deamidation can be observed in neutral regular insulin. Slow deamidation of insulin in neutral solution as well as in two different neutral suspensions was
35 reported by Schlichtkrull et al. (Handbook of Experimental Pharmacology, New Series, Vol. XXXII/2, Springer Verlag, pp. 729-777).

At higher storage temperatures significant deamidation can also be observed in neutral solution in which the hydrolytic transformation has been shown to take place in the B-chain of insulin mainly at Asn(B3) (Brange et al., Diabetologia 25 (1983), 193 (abstract)). The rate of deamidation in neutral insulin preparations varies with the formulation.

10 Whereas desamido-insulin generated in acid medium is hydrolyzed in position A21 the products formed at neutral pH are deamidated in the B-chain partly by hydrolysis of Asn(B3), partly by an α - β aspartyl rearrangement of Asn(B3) with concomitant deamidation.

15

Formation of small amounts of covalent dimerization and polymerization products of insulin during storage of neutral preparations was reported by Schlichtkrull et al. (supra) and Brange et al., Diabetologia 27 (1984), 259. In 20 neutral solutions this transformation varies with the formulation and the brand of insulin.

Insulin covalent dimerization is mainly due to a reaction of an N-terminal amino group with a carboxamide group of 25 asparagine or glutamine.

Another part of dimerization is mainly due to a reaction of aldehyde with insulin amino groups forming a bridge between two insulin molecules.

30

It is the object of the present invention to provide novel insulin analogues which are substantially less susceptible to the above described chemical transformations and degradation.

35

The objectives of this invention are achieved with the novel human insulin analogues hereinafter described.

A large number of insulin analogues have been described in the past. Märki et al. (Hoppe-Seyler's Z.Physiol.Chem., 360 (1979), 1619-1632) describe synthesis of analogues of 5 human insulin that differ from human insulin in the replacement of a single amino acid in positions 2, 5, 6, 7, 8, and 11 of the A-chain and 5, 7, 13, and 16 of the B-chain affording new insights into the intriguing structure activity relationship of insulin. Further studies modified 10 the major receptor binding area in insulin (B(22)-B(26)) to investigate the impact of such mutation on the receptor binding activity. Stability against chemical degradation and transformation was, however, not discussed.

15 By "insulin analogues" as used herein is meant a compound having a molecular structure similar to that of insulin including the disulphide bridges between Cys(A7) and Cys(B7), and between Cys(A20) and Cys(B19) and an internal disulphide bridge between Cys(A6) and Cys(A11) and with 20 insulin activity.

Summary of the invention

In its broadest aspect the present invention provides 25 insulin analogues wherein one or more asparagine residue (Asn) or glutamine residue (Gln) has been replaced by another naturally occurring amino acid residue.

The insulin analogues according to the present invention 30 can be characterized in that Asn is not present in one or more of the positions A18, A21 and B3 and/or Gln is not present in one or more of the positions A5, A15 and B4 with the proviso that when the amino acid residue in position A21 is different from Asn, then one of the amino 35 acid residues in position A15, B4 or A5 is different from Gln or one of the amino acid residues in position A18 or

B3 is different from Asn and with the proviso that when the amino acid residue in position A5 is different from Gln then one of the amino acid residues in position A15 or B4 is different from Gln or one of the amino acid residues in position A18, A21 or B3 is different from Asn.

In a more narrow aspect the present invention is related to insulin analogues wherein Asn is not present in one or more of the positions A18, A21 and B3 and/or Gln is not present in position A15 and/or B4.

Preferred insulin analogues will be such in which Asn is not present in one or more of the positions A18, A21 and B3.

15

It is considered especially important that Asn in position B3 is substituted with another amino acid residue. Preferred substituents for Asn(B3) is Asp, Gln, Ser, Thr or Gly.

20

The substituents for Gln can in principle be any naturally occurring amino acid residue with no free amide group in the side chain. Preferred substituents are Glu, Asp, His, Ser, Thr, Val, Leu, Ile, Ala, Met, Trp, Tyr and Gly, Glu and Asp being most preferred.

The substituents for Asn can in principle be any naturally occurring amino acid residue, including Gln. Preferred substituents are Glu, Asp, His, Ser, Thr, Val, Leu, Ile, Ala, Gly, Met, Trp, Tyr and Gln, Glu and Asp being most preferred.

Although Asn will preferably be substituted with an amino acid residue containing no free amide group in the side chain, a stabilization against chemical degradation might in some instances be obtained by substituting Gln for Asn. The reason for this is that Asn is considered to be more

susceptible than Gln to be deamidated, to form rearrangement products or to participate in dimerization reactions.

Due to the fact that asparagine is more susceptible to the above mentioned transformations and degradations a preferred group of insulin analogues are such in which Asn is not present in one or more of positions A18, A21 and B3.

As mentioned above it is preferred that there is no Asn in position B3. Replacement of Asn in B3 may be combined with replacement of Asn(A21) or Asn(A18). Also replacement of Asn in B3 may be combined with replacement of Asn in A(18) and A(21) or Gln in A15 and Asn in A18.

The present invention is not contemplated to be related to stabilization of native insulins such as human, pork or beef insulin, only. In the recent years a number of insulin analogues have been developed in which certain amino acid residues of human insulin have been replaced by other amino acid residues.

In European patent application No. 2148261 rapid acting insulin analogues are described in which one or more of the amino acid residues in the positions A8, A9, A10, A13, A21, B1, B2, B5, B9, B10, B12, B14, B16, B17, B18, B20, B26, B27 and B28 of human insulin are replaced by another amino acid residue. Preferred substituents are Glu and Asp. As these insulin analogues have retained Asn and Gln in positions A18 and B3 or A5, A15 and B4, respectively, they may as well be stabilized against chemical degradation according to the present invention.

Examples of such rapid acting insulin analogues being stabilized against chemical degradation are such containing an Asp or Glu in position B9, B10, B27 and/or B28.

Another group of known insulin analogues are described in European patent application No. 254,516. In these insulin analogues a basic amino acid residue has been substituted in B27 position and/or a neutral amino acid residue has been inserted in the A4, A17, B13 and/or B21 position. Furthermore Asn(A21) may be substituted by another amino acid residue in order to increase the stability of insulin solution containing the protracted insulin analogues at acid pH. The present invention, however, goes further than what is described in EP No. 254,516 suggesting substitutions of Gln and/or Asn in position A5, A15, A18, B3 and B4 as well.

The present invention is also contemplated to comprise certain derivations or further substitutions of the insulin analogues provided that such derivations or substitutions have no substantial impact on the aimed goal of the invention. It is accordingly possible to derivate one or more of the functional groups in the amino acid residues. For instance, intended to be within the scope of this invention would be per se known conversion of acid groups in the insulin molecule into ester or amide groups and conversion of alcohol groups into alkoxy groups or vice versa.

Also intended to be within the scope of this invention is addition or removal of a few amino acid residues at the N- or C-terminal ends of the A- and B-chains provided that this has no significant impact on the overall properties of the insulin analogues.

Such modifications at the ends of the A- and B-polypeptide chains may be exercised in vitro on the insulin analogues according to the present invention or by means of recombinant DNA-technology, as will be apparent for the person skilled in the art.

The novel insulin analogues according to the present invention may be prepared by altering the proinsulin gene through replacement of codon(s) at the appropriate site in the native human proinsulin gene by codon(s) encoding the
5 desired amino acid residue substitute(s) or by synthesizing the whole DNA-sequence encoding the desired insulin analogue. The gene encoding the desired insulin analogue is then inserted into a suitable expression vector which when transferred to a suitable host organism, e.g. E. coli,
10 Bacillus or yeast, generates the desired product. The expressed product is then isolated from the cells or the culture broth depending on whether the expressed product is secreted from the cells or not.

15 The novel insulin analogues may also be prepared by chemical synthesis by methods analogue to the method described by Märki et al. (Hoppe-Seyler's Z. Physiol.Chem., 360 (1979), 1619-1632). They may also be formed from separately in vitro prepared A- and B-chains containing
20 the appropriate amino acid residue substitutions, whereupon the modified A- and B-chains are linked together by establishing disulphide bridges according to known methods (e.g. Chance et al., In: Rick DH, Gross E (eds) Peptides: Synthesis - Structure - Function. Proceedings of the
25 seventh American peptide symposium, Illinois, pp. 721-728).

The insulin analogues may furthermore be prepared by a method analogue to the method described in EP patent application No. 0163529A, the disclosure of which is
30 incorporated by reference hereinto. By such a method an insulin precursor of human insulin wherein Lys^{B29} is connected to Gly^{A21} by means of either a peptide bond or a peptide chain of varying length with correctly positioned disulphide bridges is expressed and secreted by yeast and
35 then converted into human insulin by the so-called transpeptidation reaction.

Accordingly the present insulin analogues may be prepared by inserting a DNA-sequence encoding a precursor of the insulin analogue in question into a suitable yeast expression vehicle which when transferred to yeast is
5 capable of expressing and secreting the precursor of the insulin analogue in which Lys^{B29} is connected to Gly^{A21} by a peptide bond or a peptide chain with the formula I



10

wherein R is a peptide chain with n amino acid residues, n is an integer from 0 to 33 and R¹ is Lys or Arg when culturing the transformed yeast strain in a suitable nutrient medium. The precursor is then recovered from the
15 culture broth and reacted with an amino compound with the formula II



20 wherein Q is the amino acid residue which is to be inserted in the B30 position, preferably Thr, and R'' is a carboxy protecting group (e.g. methyl or tert-butyl), using trypsin or trypsin-like enzyme as a catalyst in a mixture of water and organic solvents analogously as described in US patent
25 specification No. 4,343,898 (the disclosure of which is incorporated by reference hereinto) whereupon the carboxy protecting group is removed and the insulin analogue is isolated from the reaction mixture.

30 The insulin analogues may also be prepared by a method analogue to the method described in EP patent application No. 86302133.3 the disclosure of which is incorporated by reference hereinto. By this method insulin precursors of the type having a bridge between the A- and B-chain
35 consisting of a single pair of basic amino acid (Lys, Arg) are made in yeast and then converted into insulin by an enzymatic conversion.

The present insulin analogues may be used for the preparation of novel insulin preparations with insulin activity to be substituted for human or porcine insulin in the insulin preparations heretofore known to the art. Such novel insulin preparations contain the insulin analogues according to the present invention or a pharmaceutically acceptable salt thereof in aqueous solution or suspension, preferably at neutral pH. The aqueous medium is made isotonic, for example with sodium chloride, sodium acetate or glycerol. Furthermore, the aqueous medium may contain zinc ions, buffers such as acetate and citrate and preservatives such as m-cresol, methylparaben or phenol. the pH value of the preparation is adjusted to the desired value. The insulin preparation is made sterile by sterile filtration.

The insulin preparations of this invention can be used similarly to the use of the known insulin preparations.

20

Terminology

The abbreviations used for the amino acids are those stated in J.Biol.Chem. 243 (1968), 3558. The amino acids are in the L configuration. Unless otherwise indicated, the species of insulins stated herein is human.

As used in the following text B(1-29) means a shortened B-chain of human insulin from B(1)Phe to B(29)lys and A(1-21) means the A-chain of human insulin.

The substitution(s) made in the human insulin molecule according to the practice of the invention is (are) indicated with a prefix referenced to human insulin. As an example Glu(B3) human insulin means a human insulin analogue wherein Glu has been substituted for Asn in position 3 in the B-chain. Glu(B3),B(1-29)-Ala-Ala-Lys-

A(1-21) human insulin means a precursor for the insulin analogue wherein Glu has been substituted for Asn in position 3 in the shortened B-chain (see above) and wherein the B(1-29)-chain and the A-chain (A(1-21)) are connected
5 by the peptide sequence Ala-Ala-Lys. Unless otherwise stated it is to be understood that the B(1-29) chain and A(1-21) chain are connected by disulphide bridges between A(7)Cys and B(7)Cys and between A(20)Cys and B(19)Cys, respectively, as in human insulin and that the A-chain
10 contains the internal disulphide bridge between A(6)Cys and A(11)Cys.

Detailed description

15

Genes encoding the precursors of the insulin analogue can be prepared by modification of genes encoding the corresponding human insulin precursors by site specific mutagenesis to insert or substitute with codons encoding
20 the desired mutation. A DNA-sequence encoding the precursor of the insulin analogue may also be made by enzymatic synthesis from oligonucleotides corresponding in whole or part to the insulin analogue precursor gene.

25 DNA-sequences containing a gene with the desired mutation are then combined with a suitable promoter sequence, e.g. fragments coding for the TPI promoter (TPIp) (T. Alber and G. Kawasaki, Nucleotide Sequence of the triose Phosphate Isomerase Gene of *Saccharomyces cerevisiae*. J.Mol.Applied
30 Genet. 1 (1982), 419-434), a suitable leader sequence and possible transcription termination sequence, e.g. from TPI of *S. cerevisiae* (TPI_T). These fragments provide sequences to ensure a high rate of transcription for the precursor encoding gene and also provide a presequence which can
35 effect the localization of precursor into the secretory pathway and its eventual excretion into the growth medium. The expression units are furthermore provided with a yeast

origin of replication, for instance the 2 μ origin, and a selectable marker, for instance LEU 2.

For the purpose of this invention we prepared the genes
5 encoding the insulin analogue precursor in question by
ligation of 10 oligonucleotides followed by insertion of
the gene into a yeast expression plasmid as described by
L. Thim et al., Proc.Natl.Acad.Sci. USA 83 (1986), 6766-
6770, and J. Markussen et al., Protein Engineering 1
10 (1987), 215-223.

Example 1

15

Production of Glu(A15), Asp(A18), Asp(B3) human insulin

A synthetic gene for the precursor Asp(B3)B(1-29)-Ala,Ala-
Lys-Glu(A15),Asp(A18),A(1-21) was constructed from 10
20 oligonucleotides by ligation. The oligonucleotides were
synthesized on an automatic DNA synthesizer using
phosphoramidite chemistry on a controlled pore glass
support (Beaucage, S.L. and Caruthers, M.H. Tetrahedron
Letters 22 (1981), 1859-1869). The synthetic gene was as
25 follows:

12

B1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
F	V	D	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	
HinfI Sali								NlaIV								HindIII RsaI			

5 ATTCGTCGACCAACACTTGTGCGGTTCCCACTTGGTTGAAGCTTTGTACTTGGTTTGC-
GCAGCTGGTTGTGAACACGCCAAGGGTGAACCAACTTCGAAACATGAACCAAACG-

10

20	21	22	23	24	25	26	27	28	29										
G	E	R	G	F	F	Y	T	P	K	AlaAlaLys	G	I	V	E	Q	C			
HphI			MboII			MstII, DdeI			DdeI			TagI							

15 GGTGAAAGAGGTTTCTTCTACTCCTAAGGCTGCTAAGGGTATTGTGGAACAATGC-
CCACTTTCTCCAAGAAGATGTGAGGATTCCGACGATTCCATAACAGCTTGTACG-

20

7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
C	T	S	I	C	S	L	Y	E	L	E	D	Y	C	N
RsaI					RsaI (-PvuII)					HgaI				

25 TGTACCTCCATCTGCTCCTTGTACGAATTGGAAGACTACTGCAACTAGACGCAGCC-
ACATGGAGGTAGACGAGGAACATGCTTAACCTTCTGATGACGTTGATCTGCGTCGG-

30

XbaI

CGCAGGCT
 GCGTCCGAGATC

35

Letters and numbers above the DNA-sequence indicate the corresponding amino acid residue (by means of the one letter abbreviation) and its position, respectively, in the B- and in the A-chain. The sequence AlaAlaLys is the bridge between the A- and B-chain. Also shown are the endonuclease restriction sites in the synthetic gene.

The synthetic gene was ligated to a 330 bp EcoRI-HgaI fragment from plasmid pKFN9 coding for MFa1 signal and leader sequence(1-85) and to the large EcoRI-XbaI fragment from pUC19. The construction of pKFN9 containing a HgaI

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site immediately after the MF α 1 leader sequence is described in EP patent application No. 0214826.

The ligation mixture was used to transform a competent E. coli strain (r⁻, m⁺) selecting for ampicillin resistance. Sequencing of a ³²P-XbaI-EcoRI fragment (Maxam, A. and Gilbert, W., Methods Enzymol. 65 (1980), 499-560) showed that plasmids from the resulting colonies contained the correct DNA-sequence.

10

One correct plasmid was selected for further use and cut with EcoRI and XbaI. The EcoRI-XbaI fragment was ligated to a 9.5 kb NcoI-XbaI fragment from pMT636 and a 1.4 kb NcoI-EcoRI fragment from pMT636. The construction of plasmid 15 pMT636 is described in WO patent application No. 89/01968. It contains the Schizo. pombe TPI gene (POT), the S. cerevisiae triosephosphate isomerase promoter and terminator, TPI_p and TPI_T (Alber, T. and Kawasaki, G., J.Mol.Appl.Gen. 1 (1982), 419-434).

20

The resulting plasmid encodes for the following sequence

TPI_p-MF α 1-signal-leader(1-85)-precursor gene-TPI_T

25 where MF α 1 is the S. cerevisiae MF α 1 coding sequence (Kurjan, J. and Herskowitz, I., Cell 30 (1982), 933-943) and signal-leader(1-85) means that the sequence contains the first 85 amino acid residues of the MF α 1 signal-leader sequence.

30

An S. cerevisiae (E2-7B XE11-36 a/ α , Δ tpi Δ tpi, pep 4-3/pep 4-3) was grown on YPGaL (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose, 1% lactate) to an O.D. at 600 nm of 0.6.

35

100 ml of culture was harvested by centrifugation, washed with 10 ml of water, recentrifuged and resuspended in 10

ml of a solution containing 1.2 M sorbitol, 25 mM Na₂EDTA pH = 8.0, and 6.7 mg/ml dithiotreitol. The suspension was incubated at 30°C for 15 minutes, centrifuged and the cells resuspended in 10 ml of a solution containing 1.2 M sorbitol, 10 mM Na₂EDTA, 0.1 M sodium citrate, pH = 5.8, and 2 mg Novozym^(R) 234. The suspension was incubated at 30°C for 30 minutes, the cells collected by centrifugation, washed in 10 ml of 1.2 M sorbitol and 10 ml of CAS (1.2 M sorbitol, 10 mM CaCl₂, 10 mM Tris-HCl (Tris = Tris(hydroxymethyl)aminomethan) pH = 7.5) and resuspended in 2 ml of CAS. For transformation 0.1 ml of CAS-resuspended cells were mixed with approximately 1 µg of the above described plasmid and left at room temperature for 15 minutes. 1 ml of (20% polyethylenglycol 4000, 10 mM CaCl₂, 10 mM Tris-HCl, pH = 7.5) was added and the mixture left for further 30 minutes at room temperature. The mixture was centrifuged and the pellet resuspended in 0.1 ml of SOS (1.2 M sorbitol, 33% v/v YPD, 6.7 mM CaCl₂, 14 µg/ml leucine) and incubated at 30°C for 2 hours. The suspension was then centrifuged and the pellet resuspended in 0.5 ml of 1.2 M sorbitol. Then, 6 ml of top agar (the SC medium of Sherman et al., (Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) containing 1.2 M sorbitol plus 2.5% agar) at 52°C was added and the suspension poured on top of plates containing the same agar-solidified, sorbitol containing medium. Transformant colonies were picked after 3 days at 30°C, resisolated and used to start liquid cultures.

Transformant strains were grown on YPD medium (1% yeast extract, 2% peptone (from Difco laboratories), and 2% glucose) in a 1500 liter tank at 30°C. The expressed product was isolated from the culture broth. The yield was 40.3 mg/liter.

Transpeptidation

0.2 mole (47.1 g) Thr-Met, HOAC was dissolved in DMF to give 100 ml solution, 50 ml 76.5% v/v DMF in water was added and 10 g of crude Asp(B3),B(1-29)-Ala-Ala-Lys-Glu(A15),Asp(A18)A(1-21) human insulin was dissolved in the mixture, which was thermostated at 12°C. Then 1 g of trypsin in 25 ml 0.05 M calcium acetate was added and after 24 hours at 12°C the mixture was added to 2 liter of acetone and the precipitated peptides were isolated by centrifugation and dried in vacuo. The Glu(A15),Asp(A18),Asp(B3),B30Thr-OMe human insulin was purified on a preparative HPLC column with silica-C18 as column material.

The Glu(A15),Asp(A18),Asp(B3),B30Thr-OMe human insulin was dispersed in water to 1% (w/v) and was dissolved by addition of 1 N sodium hydroxide to a pH value of 10.0. The pH value was kept constant at 10.0 for 24 hours at 25°C. The Glu(A15),Asp(A18),Asp(B3) human insulin formed was precipitated by addition of sodium chloride to about 8% (w/v), sodium acetate trihydrate to about 1.4% (w/v), and zinc acetate dihydrate to about 0.01% (w/v) followed by addition of 1 N hydrochloric acid to pH 5.5. The precipitate was isolated by centrifugation and purified by anion exchange chromatography and desalted by gel filtration. Yield: 2.40 g of Glu(A15),Asp(A18),Asp(B3) human insulin.

Example 2Preparation of Glu(A18),Glu(B3),Asp(B10) human insulin

A synthetic gene encoding a precursor Glu(B3),Asp(B10)-Ala-Ala-Lys-Glu(A18)A(1-21) was prepared as described in Example 1. The gene had the following sequence:

16

B1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
 F V E Q H L C G S D L V E A L Y L V C
 HinfI NlaIV HindIII RsaI

5 ATTCGTTGAACAACACTTGTGCGGTTCCGACTTGGTTGAAGCTTTGTACTTGGTTTGC-
GCAACTTGTGTGAACACGCCAAGGCTGAACCAACTTCGAAACATGAACCAAACG-

10

20 21 22 23 24 25 26 27 28 29 A1 2 3 4 5 6
 G E R G F F Y T P K AlaAlaLys G I V E Q C
 HphI MboII MstII, DdeI DdeI TagI

15 GGTGAAAGAGGTTTCTTCTACTCCTAAGGCTGCTAAGGGTATTGTCGAACAATGC-
CCACTTCTCCAAAGAAGATGTGAGGATTCCGACGATTCCATAACAGCTTGTACG-

20

7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
 C T S I C S L Y Q L E E Y C N
 RsaI RsaI (-PvuII) ScaI HgaI

25 TGTACCTCCATCTGCTCCTTGTAACCAATTGGAAGAGTACTGCAACTAGACGCAGCC-
ACATGGAGGTAGACGAGGAACATGGTTAACCTTCTCATGACGTTGATCTGCGTCGG-

30

XbaI

CGCAGGCT
 CGGTCCGAGATC

35

Letters and numbers above the DNA-sequence indicate the corresponding amino acid residue (by means of the one letter abbreviation) and its position, respectively, in the B- and in the A-chain. The sequence AlaAlaLys is the bridge between the A- and B-chain. Also shown are the endonuclease restriction sites in the synthetic gene.

The synthetic gene was inserted into a yeast transformant plasmid as described in Example 1. Transformation of yeast and cultivation of the transformed yeast strain was conducted as described in Example 1. Yield of the precursor was 87.3 mg/liter. The precursor was transpeptidized and

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converted into the above endproduct by the same procedure as in Example 1. Yield of Glu(A18),Glu(B3),Asp(B10) human insulin was 17.73 g.

5

Example 3Preparation of Gly(A21),Gln(B3) human insulin

A synthetic gene for the precursor Gln(B3)B(1-29)-Ala-Ala-
10 Lys-Gly(A21)A(1-21) was constructed as described in Example 1. The synthetic gene had the following sequence:

B1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
15 F V Q Q H L C G S H L V E A L Y L V C

HinfI

NlaIV

HindIII RsaI

20 ATTCGTTCAACAACACTTGTGCGGTTCCCACTTGGTTGAAGCTTTGTACTTGGTTTGC-
GCAAGTTGTTGTGAACACGCCAAGGGTGAACCAACTTCGAAACATGAACCAAACG-

25 20 21 22 23 24 25 26 27 28 29 A1 2 3 4 5 6
G E R G F F Y T P K AlaAlaLys G I V E Q C
HphI MboII MstII,DdeI DdeI TagI

30 GGTGAAAGAGGTTTCTTCTACACTCCTAAGGCTGCTAAGGGTATTGTGCGAACAATGC-
CCACTTTCTCCAAAGAAGATGTGAGGATTCCGACGATTCCCATAACAGCTTGTTACG-

35 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
C T S I C S L Y Q L E N Y C G
RsaI RsaI (-PvuII) XbaI

TGTACCTCCATCTGCTCCTTGTACCAATTGGAAAACTACTGCGGTTAAT
40 ACATGGAGGTAGACGAGGAACATGGTTAACCTTTTGATGACGCCAATTAGATC

Letters and numbers above the DNA-sequence indicate the corresponding amino acid residue (by means of the one
45 letter abbreviation) and its position, respectively, in the B- and in the A-chain. The sequence AlaAlaLys is the

18

bridge between the A- and B-chain. Also shown are the endonuclease restriction sites in the synthetic gene.

The synthetic gene was inserted into a yeast transformant 5 plasmid as described in Example 1. Transformation of yeast and cultivation of the transformed yeast strain was conducted as described in Example 1. Yield of the precursor was 19.6 mg/liter. The precursor was transpeptidized and converted into the above endproduct by the same procedure 10 as in Example 1. Yield of Gly(A21),Gln(B3) human insulin was 0.31 g.

Example 4

15 Preparation of His(A18),Ser(A21),Thr(B3) human insulin

A synthetic gene for the precursor Thr(B3)B(1-29)-Ala-Ala-Lys-His(A18),Ser(A21)A(1-21) was constructed as described in Example 1. The synthetic gene had the following 20 sequence:

19

B1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
 F V T Q H L C G S H L V E A L Y L V C
 HinfI MaeIII NlaIV HindIII RsaI
 5 ATTCGTTACCCAACACTTGTGCGGTTCCCACTTGGTTGAAGCTTTGTACTTGGTTTC-
GCAATGGGTTGTGAACACGCCAAGGGTGAACCAACTTCGAAACATGAACCAAACG-
 10
 20 21 22 23 24 25 26 27 28 29 A1 2 3 4 5 6
 G E R G F F Y T P K AlaAlaLys G I V E Q C
 HphI MboII MstII, DdeI DdeI TagI
 15 GGTGAAAGAGGTTTCTTCTACACTCCTAAGGCTGCTAAGGGTATTGTGCAACAATGC-
CCACTTCTCCTCAAAGAAGATGTGAGGATTCCGACGATTCCCATACAGCTTGTACG-
 20
 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
 C T S I C S L Y Q L E H Y C T
 RsaI RsaI (-PvuII) PstI XbaI
 25 TGTACCTCCATCTGCTCCTTGTACCAATTGGAACACTACTGCAGTTAAT
ACATGGAGGTAGACGAGGAACATGGTTAACCTTGTGATGACGTCAATTAGATC

30 Letters and numbers above the DNA-sequence indicate the
 corresponding amino acid residue (by means of the one
 letter abbreviation) and its position, respectively, in
 the B- and in the A-chain. The sequence AlaAlaLys is the
 bridge between the A- and B-chain. Also shown are the
 35 endonuclease restriction sites in the synthetic gene.

The synthetic gene was inserted into a yeast transformant
 plasmid as described in Example 1. Transformation of
 yeast and cultivation of the transformed yeast strain was
 40 conducted as described in Example 1. Yield of the precursor
 was 7.3 mg/liter. The precursor was transpeptidized and
 converted into the above endproduct by the same procedure
 as in Example 1. Yield of His(A18), Ser(A21), Thr(B3) human
 insulin was 0.79 g.

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Neutral solutions of the above insulin analogues containing phenol and glycerol were tested for stability after storage for four weeks at 37°C. As reference was used a zinc free neutral solution of human insulin and a rapid acting insulin Asp(B10) human insulin. Percent of desamido products were determined by HPLC. The results appear from the following table.

10

Table 1

<u>Tested compound</u>	<u>% desamido-insulin</u>
15 Glu(A15), Asp(A18), Asp(B3) human insulin	0.7
Gly(A21), Gln(B3) human insulin	0.3
His(A18), Ser(A21), Thr(B3) human insulin	0.7
20 Human insulin, zinc free (reference)	8.5

25 Glu(A18), Gln(B3), Asp(B10) human insulin	0.7
Asp(B10) human insulin (reference)	3.2

30 It appears from the results in Table 1 that the insulin analogues according to the present invention are markedly less deamidated than the reference compounds and therefore more stable at storage.

35 The novel insulin was tested for in vitro insulin activity with human insulin as standard by a free fat cell assay (FFC) as described by A.J. Moody et al., Hormone Metab. Res. 6 (1974), 12-16 using mouse adipocytes. The results are shown in the following table.

40

Table 2

5 Tested compound	FFC % of human insulin
Glu(A15), Asp(A18), Asp(B3) human insulin	58
10 Glu(A18), Glu(B3), Asp(B10) human insulin	67
Gly(A21), Gln(B3) human insulin	62.7
15 His(A18), Ser(A21), Thr(B3) human insulin	33.2

In vitro biological activity within the above magnitude will correspond to an in vivo biological activity of the same magnitude as that of human insulin (U. Ribel et al.,
20 Diabetes Research and Clinical Practice, Supp. 1 to vol 5, pos. 003-3, 1988).

CLAIMS

1. Insulin analogues characterized in that Asn is not present in one or more of the positions A18, A21 and B3, 5 and/or Gln is not present in one or more of the positions A5, A15 and B4 with the proviso that when the amino acid residue in position A21 is different from Asn, then one of the amino acid residues in position A15, B4 or A5 is different from Gln or one of the amino acid residues in 10 position A18 or B3 is different from Asn, and with the proviso that when the amino acid residue in position A5 is different from Gln then one of the amino acid residues in position A15 or B4 is different from Gln or one of the amino acid residues in position A18, A21 or B3 is different 15 from Asn.

2. Insulin analogues according to claim 1, wherein Asn is not present in one or more of the positions A18, A21 and B3 and/or Gln is not present in position A15 and/or B4. 20

3. Insulin analogues according to claim 1, characterized in that Asn is not present in one or more of the positions A18, A21 and B3.

25 4. Insulin analogues according to claim 1, wherein no Asn is present in position B3 and A18.

5. Insulin analogues according to claim 1, wherein no Asn is present in position B3 and A21. 30

6. Insulin analogues according to claim 1, wherein no Asn is present in position B3.

7. Insulin analogues according to claim 1, wherein no Asn 35 is present in position B3 and A21 and no Gln is present in position A15.

8. Insulin analogues according to claim 1, wherein one or more of the amino acid residue in position A18, A21 or B3 is Glu, Asp, His, Ser, Thr, Val, Leu, Ile, Ala, Met, Trp, Tyr, Gly or Gln and/or one or more of the amino acid residues in positions A5, A15 and B4 is Glu, Asp, His, Ser, Thr, Val, Leu, Ile, Ala, Met, Trp, Tyr or Gly.

9. Insulin analogues according to claim 1, wherein the amino acid residue in position B3 is Asp, Gln, Ser, Thr or Glu.

10. Insulin preparations with insulin activity, characterized in that they contain an insulin analogue according to any of the previous claims or a pharmaceutically acceptable salt thereof.

11. A process for the preparation of human insulin according to claim 1, characterized in that a yeast strain transformed with a replicable expression vehicle comprising a DNA-sequence encoding a precursor of the insulin analogue in which Lys^{B29} is connected to Gly^{A1} by a peptide bond or a peptide chain with the formula I



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wherein R is a peptide chain with n amino acid residues, n is an integer from 0 to 33 and R¹ is Lys or Arg, is cultured in a suitable nutrient medium, the precursor is recovered from the culture broth and reacted with an amino compound with the formula II



wherein Q is the amino acid residue which is to be inserted in the B30 position, preferably Thr, and R'' is a carboxy protecting group, using trypsin or a trypsin-like enzyme as a catalyst in a mixture of water and organic solvents

whereafter the carboxy protecting group is removed by hydrolysis.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK89/00117

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC ₄		
C 07 K 7/40, A 61 K 37/26, C 12 P 21/02, C 12 N 15/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
IPC 4	A 61 K 37/26; C 07 K 7/40, /42; C 12 N 15/00; C 12 P 21/02 .../...	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
SE, NO, DK, FI classes as above.		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Chemiker-Zeitung, 100. Jahrgang (1976) Nr.3 Rolf Geiger, "Chemie des Insulins", p 111-129, see pages 120 and 126	1-4,8,10
X	SE, B,451 461 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 12 October 1987 see page 36 & BE, 867424 LU, 79714 NL, 7805591 FR, 2392033 DE, 2822568 JP, 54049387 GB, 1565190 CA, 1105402 CA, 1109813 CA, 1112195 AU, 521903 AT, 371836 SE, 7806086 .../...	1,2,8
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
1989-08-09	1989-08-14	
International Searching Authority	Signature of Authorised Officer	
Swedish Patent Office	Elisabeth Carlborg	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	CA, 1156166 SE, 8305327 SE, 8305328 SE, 8305329 CH, 642680 US, 4440859 US, 4264731 SE, 455794 AT, 386607 AT, 386608 AT, 386609 US, 4652525 US, 4363877 US, 4407948 CA, 1157406 US, 4447538	
A	DE, A1, 2 536 040 (HOECHST AG) 24 February 1977 see page 2	1-9
A	US, A, 4 343 898 (JAN MARKUSSEN) 10 August 1982	11

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

II Fields searched (cont)

US C1 260:112.7; 424:178; 435:70,71;
514:3,4; 530:303-305

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim numbers _____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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